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Toxicology 151 (2000) 45–54

TOXICOLOGY

www.elsevier.com/locate/toxicol

Cadmium-induced elevations in the gene expression of the regulatory subunit of γ-glutamylcysteine synthetase in rat lung and alveolar epithelial cells

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Received 28 May 2000; accepted 6 July 2000

Abstract

The controlled step in de novo glutathione (GSH) synthesis is catalyzed by γ-glutamylcysteine synthetase (γ-GCS), a dimeric enzyme consisting of a heavy catalytic subunit (γ-GCS-HS) and a light regulatory subunit (γ-GCS-LS). We have previously reported that exposure to cadmium (Cd) induces pulmonary γ-GCS-HS mRNA and protein, and that these alterations are accompanied by increases in GSH synthesis and its steady-state level. The current study was designed to test the hypothesis that Cd exposure also up-regulates the expression of the regulatory γ-GCS subunit. By using northern blotting, we have demonstrated that a single Cd aerosol exposure of adult male Lewis rats results in time- and dose-dependent increases in pulmonary levels of γ-GCS-LS mRNA. Transcripts of γ-GCS-LS in rat lung are maximally elevated (8-fold) 2 h following Cd inhalation exposure and remain significantly higher than air controls at 24 h. This response is highly correlated with Cd dose, ranging from 0.9 to 5 mg Cd per m^3, and with lung Cd burden. We also observed Cd-induced up-regulation of γ-GCS-LS mRNA expression in alveolar epithelial cells exposed to Cd in vitro, either acutely or after repeated passaging in Cd-containing medium. The magnitude of the γ-GCS regulatory subunit induction observed in Cd-treated cells was approximately five times greater than the induction of the catalytic subunit. These modifications in the expression of γ-GCS subunits may offer protection from Cd toxicity.

Keywords: Cadmium; Lung; Alveolar epithelial cells; Gene expression; γ-Glutamylcysteine synthetase

1. Introduction

An extensive use of cadmium (Cd) in a variety of industrial processes has led to an increased environmental burden of this heavy metal. It is also released in the environment through burning of fossil fuels and refining of metal ores. Exposure of humans and animals to Cd has been reported to produce multi-organ toxicity (Shukla and Singhal, 1984). Inhalation of Cd has been implicated in the development of emphysema and pulmonary fibrosis, and, recently, this metal has been desig-

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0300-483X/00/$ - see front matter
PII: S0300-483X(00)00263-8

nated as a human lung carcinogen as well (IARC, 1993). The exact mechanism by which Cd produces adverse biological effects is not known at present.

Previous studies in our laboratory have shown that pulmonary adaptation develops in animals repeatedly exposed to Cd aerosols (Hart, 1986). These animals show tolerance to subsequent acute exposure to Cd and cross-tolerance to lethal doses of oxygen (Hart et al., 1989, 1990). A number of protective agents likely contribute to this observed pulmonary resistance, including the thiol-rich, metal-binding protein, metallothionein and glutathione (GSH), a sulfhydryl-containing tripeptide (Hart, 2000; Hart et al., 1996).

GSH is synthesized by the consecutive action of two enzymes, γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase. The first enzyme in this sequence, γ-GCS, catalyzes the rate-limiting reaction of GSH biosynthesis and consists of two subunits, a heavy (~72 kDa) catalytic subunit (γ-GCS-HS) and light (~28 kDa) regulatory subunit (γ-GCS-LS). While γ-GCS-HS is responsible for all the catalytic activity, γ-GCS-LS has been shown to reduce the K_m for glutamate and increase the K_i for GSH, the feed-back inhibitor of the enzyme reaction (Huang et al., 1993). The γ-GCS subunits are encoded by separate genes located on different chromosomes. Both genes are subject to transcriptional control. Differential as well as coordinate induction of the γ-GCS subunits have been reported (Cai et al., 1997; Galloway et al., 1997; Tian et al., 1997). Moreover, the patterns of induction for these two genes appear to vary as a function of the stimulus, cell type, and organ system (Gipp et al., 1995; Dahl and Mulcahy, 2000).

Our laboratory has recently reported that exposure of rats to Cd aerosols induces the expression (mRNA and protein) of the catalytic γ-GCS heavy subunit. This response is accompanied by increases in pulmonary GSH synthetic rate as well as elevations in lung GSH levels (Shukla et al., 2000). Immunolocalization and in situ hybridization studies showed preferential induction of γ-GCS-HS in type II epithelial cells, considered to be stem cells of the alveolar epithelium. An established rat alveolar epithelial cell line, with morphological and biochemical characteristics similar to type II pneumocytes, has also been shown to exhibit similar induction of γ-GCS-HS expression following either acute or chronic in vitro exposure to Cd (Hart et al., 1999; Shukla et al., 1999; Eneman et al., 2000).

In contrast, virtually, nothing is known about the effects of the heavy metal Cd on pulmonary steady-state mRNA levels of the light subunit of γ-GCS. Since the light subunit plays such a critical regulatory role in governing the overall function of this enzyme, we considered it important to investigate how its gene regulation is affected by exposure to Cd. The goals of the current study were to, (1) test the hypothesis that, in vivo and in vitro Cd exposure also induces the pulmonary expression of the regulatory, light subunit of γ-GCS, (2) determine whether changes in steady-state levels of γ-GCS-LS mRNA vary as a function of time and Cd dose, (3) ascertain whether Cd exposure results in coordinate induction of both the regulatory and the catalytic subunit transcripts of γ-GCS, and (4) compare the ratio of γ-GCS-LS and γ-GCS-HS expression in alveolar epithelial cells following Cd exposure.

2. Materials and methods

2.1. Chemicals and supplies

Atomic absorption standards and cadmium acetate were procured from Fisher (Fair Lawn, NJ). Nitric acid of Ultrex grade was purchased from Baker Chemical Corporation (Philipsburg, NJ), Ham's F-12 nutrient mixture, glutamine, penicillin, streptomycin, trypsin, and versene were obtained from Gibco-BRL (Grand Isle, NY). Newborn serum was supplied by JRH Biosciences (Lenexa, KS). All other chemicals, unless otherwise stated, were purchased from Sigma Chemical Company (St Louis, MO). The chemicals used for northern blot analysis were of molecular biology grade. Sephadex quick spin columns were purchased from Boehringer Mannheim (Indianapolis, IN).

2.2. Animals and treatment

Male Lewis rats, weighing 180–190 g, were purchased from Harland Sprague–Dawley (Indianapolis, IN). Rats were housed in a room maintained at 26–28°C and ~50% humidity with a light/dark cycle of 12 h. Animals had a free access to food and water. A nose-only inhalation facility, as described previously by Hart (1986), was used to expose rats to the Cd aerosol or to air for 3 h. The Cd aerosol was generated from an aqueous solution of 0.15 M cadmium acetate with a 3-jet nebulizer operated at 20 psi and 7 l/min (BGI Inc., Waltman, MA). This aerosol was diluted with air and passed through a tube furnace heated at 200°C. This process converted ~80 ± 5% of the Cd in the aerosol into an insoluble oxide. Air samples in the laminar flow chamber were collected on 0.45-μm field filters (Millipore Corp., Bedford, MA) and the Cd presence was determined using a Perkin–Elmer-2380 atomic absorption spectrophotometer. The Cd aerosol had a mass median aerodynamic diameter of 0.8 μm.

2.3. Tissue processing

At predetermined times following Cd inhalation, rats were anesthetized with 50-mg pentobarbital per kg (ip) and exsanguinated by cardiac puncture. Lungs were then removed, weighed and snap-frozen in liquid nitrogen. While still immersed in liquid nitrogen, lungs were broken into small pieces and were subsequently stored at −70°C until needed for analysis.

2.4. Cell culture and treatment

An alveolar epithelial cell line, derived originally from the lungs of an adult Fisher-344 rat, was supplied by N.F. Johnson from Lovelace Inhalation Toxicology Research Institute (Albuquerque, NM). These cells are non-transformed and have been shown to resemble type II pneumocytes, both morphologically and biochemically (Li et al., 1983). Cultures were routinely grown at 37°C in an atmosphere of 5% CO_2/95% air in F-12 medium, containing 10% newborn calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and were passaged weekly. For acute Cd exposures, cultures were first grown to ~80% confluence. These cultures were made quiescent by incubation for 24 h in medium containing 2% serum prior to the addition of $CdCl_2$ and subsequent RNA extraction. In experiments involving chronic Cd exposure, cells were passaged repeatedly in complete medium containing 10 μM $CdCl_2$. RNA was subsequently extracted from these cultures when they reached confluence following the fifth passage.

2.5. Probes for northern blot hybridization

Plasmids used for studying γ-GCS expression were generously provided by T.J. Kavanagh (University of Washington, Seattle, WA). Plasmid pCR3.1-mGlclr contains the protein-coding region of cDNA for regulatory subunit of mouse γ-GCS-LS cloned into the vector, pCR3.1 (Invitrogen, Portland, OR). Plasmid pCRII-MGS-Z was constructed by sub-cloning a 390 bp fragment of mouse heavy chain γ-GCS (γ-GCS-HS cDNA) into pCRII plasmid (Invitrogen, Portland, OR), as described previously (Hart et al., 1999; Shukla et al., 2000). Following amplification and purification, inserts were cut from pR3.1-mGlclr and pCRII-MGCS-Z with Pme (New England Biolabs Inc., Beverly, MA) and *Eco*R1 (Gibco-BRL, Grand Isle, NY), respectively. A cytoplasmic 7S RNA (pA6) served as an internal standard (Cook and Chiu, 1986). Purified cDNA probes were subsequently labeled with [α-^{32}P]dCTP (6000 Ci/mmol) purchased from Amersham (Arlington Heights, IL), using a random prime labeling procedure (Gibco-BRL, Grand Isle, NY).

2.6. RNA isolation and northern blot analysis

Total RNA from lung tissue and cells was isolated by using STAT-60 RNAzol (Tel-test, Inc., Friendwood, TX), and subsequent chloroform extraction procedure. Northern blotting protocols for γ-GCS-HS (Hart et al., 1999) and cytoplasmic 7S (Cook and Chiu, 1986) were performed using Zeta Probe GT nylon membranes (Bio-Rad, Richmond, CA). Conditions used for γ-GCS-LS hybridization were identical to the pro-

tocol used for γ-GCS-HS. Hybridization signals for γ-GCS-LS mRNA and γ-GCS-HS mRNA were detected using Biomax MS autoradiography films (Eastman Kodak Co., Rochester, NY), quantitatively analyzed with a GS-250 PhosphorImager (Bio-Rad, Hercules, CA), and normalized for differences in RNA application using 7S RNA as an internal standard (Cook and Chiu, 1986). Results are presented as counts per 25 μg total RNA.

2.7. Lung cadmium analysis

Lung tissue weighing ~200 mg was digested in nitric acid (Ultrex grade) for 24 h at 55°C and analyzed for Cd, using a Perkin Elmer-2380 spectrophotometer equipped with air–acetylene flame. The data are expressed as μg Cd per whole lung.

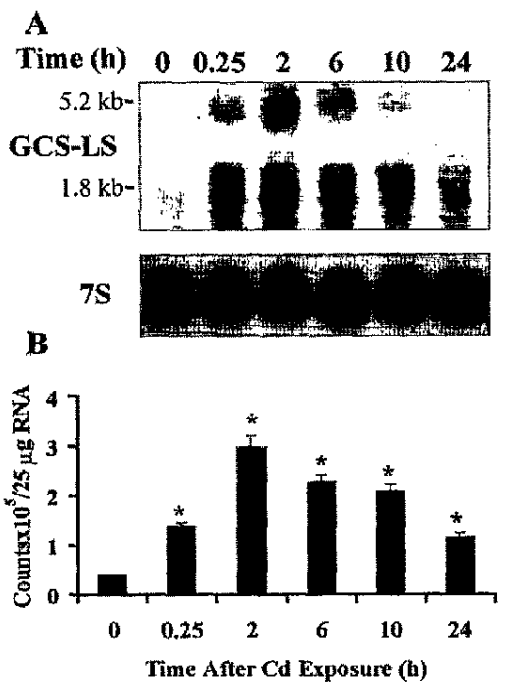

Fig. 1. Time-dependent changes in steady-state levels of rat lung γ-GCS-LS mRNA following a single 3-h aerosol exposure to 2.44 mg Cd per m³. (A) Film autoradiograms of northern blots for γ-GCS-LS and 7S RNA; (B) quantitative hybridization data, normalized using the 7S RNA as an internal standard. PhosphorImager counts (pixel units) were derived using 25-μg total RNA. Each bar represents the mean ± S.E.M. using four independent samples; *, denotes significantly different ($P < 0.05$) from the value at $t = 0$.

2.8. Statistical analysis

Statistical comparisons were performed by unpaired t-tests or analysis of variance using Student–Newman–Keuls to adjust for multiple comparisons. When variability between groups differed, logarithmic transformations of data were used. The level of significance was established at $P < 0.05$.

3. Results

3.1. Cd-induced expression of γ-GCS-LS in rat lung

To determine if Cd induced the expression of pulmonary γ-GCS-LS, northern blot analysis was performed on total RNA extracted from lungs of animals following a single 3-h inhalation exposure to an atmosphere containing 2.4 mg Cd per m³. The film autoradiogram in Fig. 1A shows that, basal levels of pulmonary γ-GCS-LS mRNA at $t = 0$ are low and that expression levels increase in a time-dependent manner following Cd inhalation exposure. Two distinct transcripts were found to hybridize with the radiolabeled γ-GCS-LS probe. The 1.8 kb γ-GCS-LS transcript appeared to be more prominent than the 5.2 kb transcript. In contrast to Cd-induced increases in γ-GCS-LS message levels, no change was observed in the expression of the 7S RNA housekeeping gene (Fig. 1A), making it suitable as an internal standard. As shown in Fig. 1B, steady-state levels of γ-GCS-LS mRNA were significantly increased (3.7-fold) above control ($t = 0$) as early as 15 min following Cd exposure. Maximum induction of γ-GCS-LS mRNA was achieved at 2-h post-exposure, at which time, expression was elevated 8.1-fold. Message levels at 6, 10, and 24 h, albeit lower than at $t = 2$-h post-exposure were 6.2-, 5.7-, and 3.1-fold higher than transcript abundance observed at $t = 0$ (Fig. 1B).

Relative changes in steady-state γ-GCS-LS mRNA levels as a function of Cd aerosol concentration were then examined. In this experiment, RNA was extracted from rat lungs 6 h after a single (3-h) exposure to 0, 0.9, 1.6, 3.5, or 5.0 mg

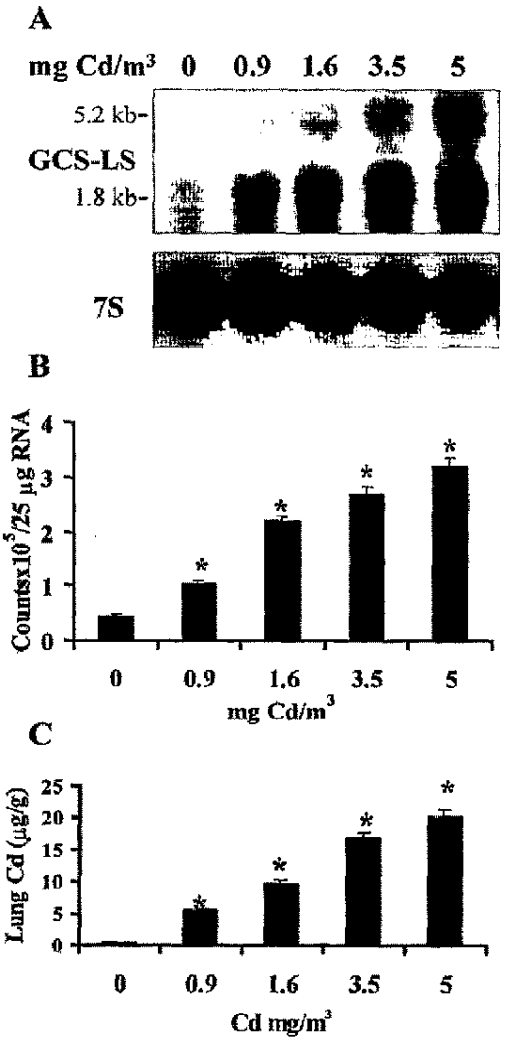

Fig. 2. Dose-dependent effect of Cd inhalation on the steady-state levels of γ-GCS-LS mRNA and on lung Cd burden. Animals were exposed for 3 h to aerosols containing 0, 0.9, 1.6, 3.5, or 5.0 mg Cd per m^3 and were subsequently killed 6-h post-exposure. (A) Film autoradiograms of northern blots for γ-GCS-LS mRNA and 7S RNA; (B) quantitative hybridization data for γ-GCS-LS mRNA, normalized using 7S RNA as an internal standard. PhosphorImager counts (pixel units) were derived using 25-µg total RNA; (C) lung Cd burden. Each bar represents the mean ± S.E.M., using four independent samples; *, denotes significantly different ($P < 0.05$) from levels in animals exposed to air (0 mg Cd per m^3).

Cd per m^3. As Fig. 2 shows, a significant dose-dependent linear increase in γ-GCS-LS was observed over the range of Cd concentrations

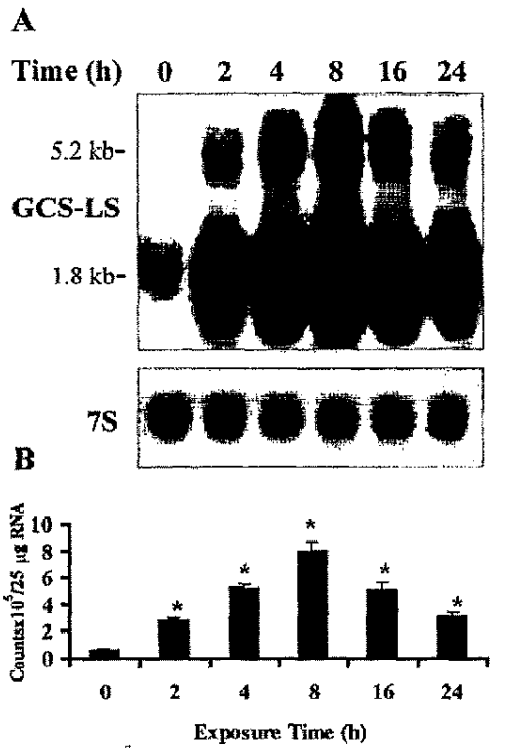

Fig. 3. Northern hybridization study describing the kinetics of Cd-induced alterations in steady-state levels of γ-GCS mRNA. Total RNA was extracted from alveolar epithelial cells at 0, 2, 4, 8, 16, and 24 h after the addition of 10 µM $CdCl_2$. (A) Autoradiogram of a representative northern blot for γ-GCS-LS mRNA and 7S RNA; (B) quantitative hybridization data, normalized for differences in RNA loading using 7S as an internal control. PhosphorImager counts (in pixel units) were derived using 25-µg total RNA. Each bar represents the mean ± S.E.M. using three independent samples; *, denotes significantly different ($P < 0.05$) from the value at $t = 0$.

evaluated. Moreover, correlations of Cd concentration with (a) γ-GCS-LS mRNA ($r = 0.95$) and with (b) lung Cd burden ($r = 0.98$) were highly significant ($P < 0.001$, Fig. 3).

3.2. Cd-induced expression of γ-GCS-LS in rat alveolar epithelial cell cultures

Northern analysis was performed to determine the relative alterations in γ-GCS-LS mRNA in rat alveolar epithelial cells, exposed acutely or chronically to Cd in vitro. First, we evaluated the time-dependent changes in γ-GCS-LS mRNA

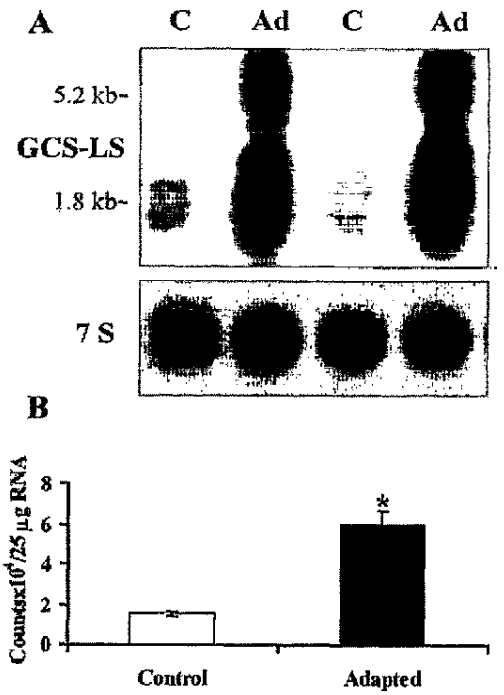

Fig. 4. γ-GCS-LS gene expression in non-adapted and Cd-adapted alveolar epithelial cells. (A) Representative autoradiogram of a northern blot for γ-GCS-LS mRNA and 7S RNA. C, control, non-adapted cells. Ad, adapted cells passaged five times in medium containing 10 μM $CdCl_2$; (B) quantitative hybridization data for γ-GCS-LS mRNA, normalized for RNA loading using 7S RNA as an internal standard. Hybridization counts, obtained with a PhosphorImager, were expressed as pixel units per 25-μg total RNA. Each bar represents the mean ± S.E.M., where $N = 3$; *, denotes significantly different ($P < 0.05$) from values in control, non-adapted cells.

expression following the addition of 10 μM $CdCl_2$ to the culture medium. The results of this kinetic study are shown in Fig. 4. At 2 h, following Cd addition, the expression level for γ-GCS-LS rose significantly at a level 4.5-fold greater than in control cells. Message levels exhibited a significant linear upward trend for 8 h following Cd addition. At this point, Cd-induced expression of γ-GCS-LS peaked at a level 12.8-fold control. Even at 24 h, message levels were still 5.1-fold higher than in non-exposed cells. In a second set of experiments, we compared the steady-state γ-GCS mRNA level in alveolar epithelial cells that were passaged a total of five times in culture medium containing 10 μM $CdCl_2$ with the γ-GCS-LS expression level in cells with no prior exposure to Cd. As shown in Fig. 4, the alveolar epithelial cell culture, which was adapted by repeated passaging in Cd-containing medium, had an approximately 4-fold higher level of γ-GCS-LS mRNA compared with non-adapted control cells.

3.3. Comparison of γ-GCS-LS and γ-GCS-HS expression following in vitro Cd exposure

Quiescent rat alveolar epithelial cells were treated for 8 h with 0, 5, or 10 μM $CdCl_2$. The total cellular RNA was subsequently analyzed by northern blotting and the relative induction levels for LS and HS of γ-GCS were compared. The results, shown in Fig. 5, indicate that Cd-induced expression of both γ-GCS subunits is coordinate and dose-dependent. Exposure of cells to 5 and 10 μM $CdCl_2$ resulted in, (a) a 12.5- and a 17.1-fold increase in γ-GCS-LS mRNA, respectively, and (b) a 2.4- and a 3.1-fold increase in γ-GCS-HS mRNA, respectively. Induction of γ-GCS-LS mRNA by Cd was more than five times greater than that of γ-GCS mRNA.

4. Discussion

Glutathione is the most abundant low-molecular weight thiol in mammalian cells. As such, it plays a major role in cellular detoxification processes by providing the cell with multiple defenses against reactive oxygen species and also against their toxic products (Hayes and McLellan, 1999). GSH also has a high affinity for heavy metals and, as a result, constitutes the first line of defense against Cd toxicity (Singhal et al., 1987). In some of our earlier studies we found that GSH accumulates in the lungs of animals following repeated inhalation of Cd (Hart et al., 1989, 1990) and in alveolar epithelial cells exposed to Cd in vitro (Gong and Hart, 1997). Although multiple factors can contribute to Cd-induced elevations in GSH concentrations, we considered that the regulation of γ-GCS expression was of prime importance (Griffith, 1999; Wild and Mulcahy, 2000).

γ-GCS catalyzes the rate-limiting, controlled step in de novo GSH synthesis. This enzyme is composed of a heavy and a light subunit encoded by genes on different chromosomes. Although the rat heavy subunit is represented by a single mRNA, hybridization experiments consistently show two transcripts coding for the light subunit (Gipp et al., 1995; Griffith, 1999). The heavy subunit (γ-GCS-HS) is responsible for all of the catalytic activity and it is subject to feedback inhibition by GSH. Kinetic properties of the catalytic subunit are, however, significantly affected by its association with the light or regulatory subunit (γ-GCS-LS). Experiments using purified subunits of γ-GCS (Huang et al., 1993; Griffith, 1999) have demonstrated that the availability of the light subunit significantly enhances the catalytic efficiency of the heavy subunit and reduces its sensitivity to the inhibitory action of GSH (Huang et al., 1993). The importance of both γ-GCS subunits is also indicated by the finding that higher GSH concentrations occur in cells transfected with a combination of the regulatory and catalytic cDNAs than with the catalytic subunit cDNA alone (Mulcahy et al., 1995).

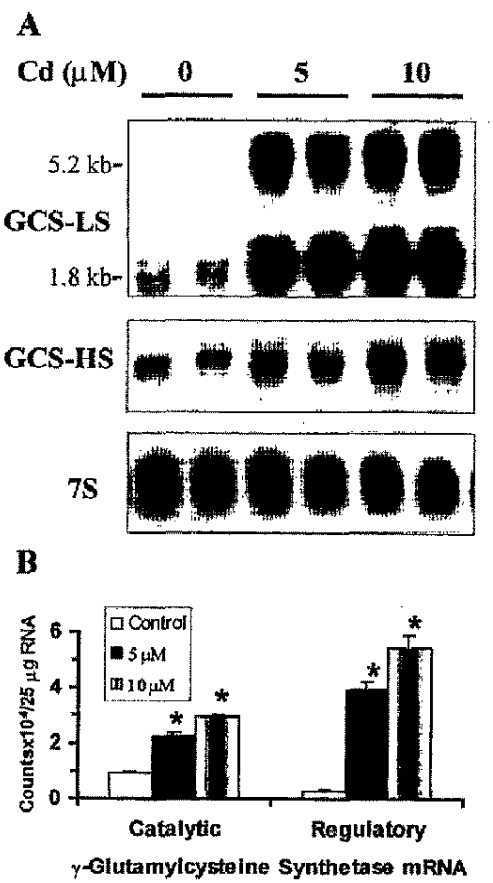

Fig. 5. Comparison of steady-state mRNA levels of γ-GCS catalytic subunit (γ-GCS-HS) and γ-GCS regulatory subunit (γ-GCS-LS) in alveolar epithelial cells exposed to 0, 5, or 10 μM $CdCl_2$ for 8 h. (A) Representative autoradiogram of a northern blot for γ-GCS-LS mRNA, γ-GCS-HS mRNA, and 7S RNA; (B) quantitative hybridization data, obtained with a PhosphorImager, were normalized for differences in RNA application using 7S RNA as an internal control. Each bar represents the mean ± S.E.M., where $N = 3$; *, significantly different ($P < 0.05$) from γ-GCS-LS mRNA or γ-GCS-LS mRNA in control cells (0 μM $CdCl_2$).

Our laboratory has now completed a series of studies related to the effects of Cd on the expression of the catalytic γ-GCS subunit in the lung (Hart et al., 1999; Shukla et al., 1999; Eneman et al., 2000; Shukla et al., 2000). In one of these studies, we demonstrated that inhalation exposure of rats to CdO aerosols caused up-regulation in pulmonary levels of γ-GCS-HS mRNA and γ-GCS protein (Shukla et al., 2000). Immunohistochemical and in situ hybridization studies further revealed that these changes in catalytic subunit expression following Cd inhalation were most prominent within the alveolar epithelial compartment of the lung, most likely in type II alveolar epithelial cells. Pulmonary elevations in γ-GCS-HS mRNA levels in response to Cd inhalation were accompanied by higher GSH levels and an increased GSH synthetic rate (Shukla et al., 2000). Elevations in γ-GCS-HS mRNA in Cd-resistant human lung tumor cells (Hatcher et al., 1995) and in rat alveolar epithelial cells exposed in vitro to $CdCl_2$, either acutely (Hart et al., 1999; Shukla et al., 1999) or sub-chronically, (Eneman et al., 2000) have also been reported.

The availability of a cDNA probe which is specific for detecting mRNA levels for the γ-GCS-LS has now enabled us to test whether Cd also stimulates the transcriptional activation of the regulatory subunit in the lung. These findings are presented in this report. To our knowledge, we are the first to show that Cd, administered by inhalation to the respiratory tract, also up-regu-

lates the expression of the pulmonary γ-GCS regulatory subunit. Similar to our previous findings with the catalytic subunit (Shukla et al., 2000), induction of the regulatory subunit of γ-GCS by Cd occurs in a time- and dose-dependent manner and it is highly correlated with Cd lung burden. Moreover, the induction kinetics for both subunits is similar. Significant increases in message levels occur within 15 min following the Cd aerosol treatment. Maximum transcript levels for both subunits are achieved by 2-h post-exposure to Cd. Message levels for both subunits then slowly decline at times thereafter at approximately the same rates. However, even 24 h after Cd treatment, the transcripts for both γ-GCS genes are still higher in the lungs of Cd-treated animals than in air controls.

We have also demonstrated in this report that coordinate induction of both subunits occurs when rat alveolar epithelial cells are exposed to Cd in vitro. This was true for cells treated acutely with Cd and for cells repeatedly passaged in medium containing Cd. However, even where the two genes appear to be co-regulated, subtle differences in gene regulation have still been noted. Specifically, we have observed differences in the magnitude of induction of these two genes in response to Cd. Induction for the regulatory gene was approximately five times higher than for the catalytic gene. This difference was due to both increased Cd-induced expression and decreased basal levels of mRNA. Whether the additional transcripts are translated is not known at present.

Several other investigators have also reported that the transcription of γ-GCS-HS and γ-GCS-LS does not appear to be tightly coordinated. Gipp et al. (1995) analyzed normal human tissue to determine basal mRNA levels for the heavy and light subunits of γ-GCS. These investigators discovered that the absolute amounts of basal catalytic mRNA and regulatory mRNA vary considerably from tissue to tissue. Differences in the induced regulation of γ-GCS subunit genes have also been reported. For example, Liu et al. (1998) demonstrated that both γ-GCS subunits could be induced in lung epithelial cells by 4-hydroxy-2-nonenal. However, similar to our findings, they found that the light subunit was induced to a greater extent than the heavy subunit. In other studies, Dahl and Mulcahy (2000) measured regulatory and catalytic gene expression in cell lines derived from liver, kidney, skeletal muscle, and lung tumor that were treated with pyrrolidine dithiocarbonate (PDTC), phenylethyl isothiocyanate, or β-napththoflavone. Both subunits were induced in liver cells by all three chemicals, but the regulatory subunit was induced to a greater extent than the catalytic subunit. In contrast, no induction of either subunit occurred in the other cell lines except in response to PDTC, and then only the expression of the regulatory subunit was increased.

The physiological significance of these differences in subunit expression is not currently understood. Variation in the relative amounts of the heavy and light subunits may represent an additional means by which γ-GCS activity can be regulated. This concept is supported by studies by Tipnis et al. (1999) who demonstrated that the addition of recombinant γ-GCS-LS to HeLa cell extracts increased γ-GCS activity. In other experiments, they found that γ-GCS activity and GSH levels were increased in COS-1 cells following transient transfection with a regulatory subunit cDNA.

The biochemical signals by which Cd initiates the series of events that eventually culminate in the transcriptional regulation of the γ-GCS subunit genes also remain to be elucidated. The involvement of reactive oxygen species (ROS) has been proposed as one possible signaling mechanism which could initiate γ-GCS expression (Talalay et al., 1995). The Cd ion is unable to catalyze Fenton/Haber–Weiss reactions which generate ROS (Kasprzak, 1995). However, experiments conducted by Yang et al. (1997), using oxidant-sensitive fluorescent probes, have shown that Cd-induced increases in intracellular ROS formation can occur in a time- and dose-dependent fashion. Thiol modification of key regulatory proteins may represent another signaling mechanism (Talalay et al., 1995). Such modifications could result from direct binding of Cd with protein sulfhydryl groups. Alternatively, the increased levels of oxidized glutathione, which accumulate in lung cells following Cd exposure (Hart

et al., 1999; Shukla et al., 1999, 2000), could participate in the formation of protein-bound glutathione conjugates.

Several putative transcription initiation sites have been identified in the 5′ flanking regions of both γ-GCS subunit genes. These cis-acting factors include antioxidant response elements (AREs), multiple activator protein-1 (AP-1) or AP-1-like binding sites, and metal response elements (MREs). A consensus sequence for nuclear factor-κB (NF-κB) is only present in the promoter of the catalytic subunit (Wild and Mulcahy, 2000). The exact sequence of nucleotides which mediates basal or Cd-induced γ-GCS-HS and γ-GCS-LS expression is presently unknown.

Because steady-state mRNA levels, corresponding to the γ-GCS subunit genes, are known to vary in different tissues and cells and with different inducing agents, distinct sets of transcription factors are also likely to participate in the regulation of basal and inducible expression. The prevailing evidence suggests that members of the AP-1 family play a role (Wild and Mulcahy, 2000). We have previously shown that exposure of alveolar epithelial cells to Cd enhances the expression of AP-1, as demonstrated by electrophoretic mobility shift assays (Hart et al., 1999; Shukla et al., 1999). However, it is possible that other transacting factors may also participate including, MTF-1, the metal-responsive transcriptional activator (Gunes et al., 1998) or members of the NF-E2 and Maf families (Wild and Mulcahy, 2000).

In summary, this study has demonstrated for the first time that Cd up-regulates the expression of the regulatory light subunit of γ-GCS in lungs of animals following Cd inhalation and in alveolar epithelial cells exposed to Cd in vitro. Although the kinetics of induction for the regulatory and catalytic subunits is similar, the γ-GCS-LS gene is induced by Cd to a greater extent than the γ-GCS-HS gene. This difference is due to lower basal and higher inducible light subunit expression and may be cell-specific. An increase in expression of γ-GCS-LS, together with γ-GCS-HS, may be essential for the observed increases in pulmonary GSH levels following Cd exposure and likely plays a major role in the Cd adaptive process.

Acknowledgements

This research was supported by a grant from the National Institute of Environmental Health Sciences (ES 08991). Our appreciation is extended to Dr Pamela Vacek for statistical analyses. During the course of these studies, Dr Girja Shukla was on leave from the Industrial Toxicology Research Center, Lucknow, India.

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